Chloride conductance in mouse muscle is subject to post-transcriptional compensation of the functional Cl^- channel 1 gene dosage

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1. In mature mammalian muscle, the muscular chloride channel ClC-1 contributes about 75% of the sarcolemmal resting conductance (G_m). In mice carrying two defective alleles of the corresponding *Clc1* gene, chloride conductance (G_{Cl}) is reduced to less than 10% of that of wild-type, and this causes hyperexcitability, the salient feature of the disease myotonia. Potassium conductance (G_K) values in myotonic mouse muscle fibres are lowered by about 60% compared with wild-type.
2. The defective Clc^{adr} allele causes loss of the 4·5 kb ClC-1 mRNA. Mice heterozygous for the defective $Clc1^{adr}$ allele contain about 50% functional mRNA in their muscles compared with homozygous wild-type mice.
3. Despite a halved functional gene dosage, heterozygous muscles display an average G_{Cl} which is not significantly different from that of homozygous wild-type animals. The G_K values in heterozygotes are also indistinguishable from homozygous wild-type animals.
4. These results indicate that a regulatory mechanism acting at the post-transcriptional level limits the density of ClC-1 channels. G_K is probably indirectly regulated by muscle activity.

Hereditary myotonias are muscle diseases characterized by cramp-like aftercontractions upon voluntary movements, caused by a hyperexcitability of the sarcolemma. They may either be due to dominant mutations in the gene for the muscular sodium channel Skm-1 (for review see Lehmann-Horn & Rüdel, 1996) or dominant Thomsen- or recessive Becker-type mutations in the gene for the major muscular chloride channel ClC-1 (Koch *et al.* 1992; for review see Lehmann-Horn & Rüdel, 1996). This latter finding was based on the discovery of myotonia and lowered sarcolemmal total and chloride conductances (G_m and G_{Cl}, respectively) (Brinkmeier & Jockusch, 1987*a*; Mehrke, Brinkmeier & Jockusch, 1988) in a mouse mutant affected by the recessive mutation 'arrested development of righting response' (*adr*; Watkins & Watts, 1984). In this mouse mutant, the chloride channel gene *Clc1* on chromosome 6 was shown to be functionally and structurally affected and to be identical to the *adr* myotonia gene (Steinmeyer *et al.* 1991*a*). Myotonia mutations in the mouse, spontaneous or induced, are recessive and allelic with each other (Jockusch, Bertram & Schenk, 1988; Adkison, Harris, Lane & Davisson, 1989). Hence, all myotonias in the mouse are chloride channel diseases of the recessive Becker type. We have molecularly analysed alleles $Clc1^{adr}$ (retroposon insertion; Steinmeyer *et al.* 1991*a*; Schnülle, Antropova, Wedemeyer, Jockusch & Bartsch, 1997), $Clc1^{adr\text{-}mto}$ (stop codon; Gronemeier, Condie, Prosser, Steinmeyer, Jentsch & Jockusch, 1994), and $Clc1^{adr\text{-}K}$ (missense mutation; Gronemeier *et al.* 1994). Using an antibody against a ClC-1 peptide that reacts with the ClC-1 protein in frozen sections, Gurnett, Kahl, Anderson & Campbell (1995) showed the ClC-1 channel to be present on the sarcolemma and demonstrated its absence from myotonic muscle of the homozygous $Clc1^{adr\text{-}mto}$ mutant, in which the ClC-1 polypeptide is truncated close to its N-terminus (Gronemeier *et al.* 1994).

We have shown that *Clc1* gene dosage is critical for the excitability of neonate mouse muscle (Wischmeyer, Nolte, Klocke, Jockusch & Brinkmeier, 1993) during a period when ClC-1 mRNA levels in rodents are still low (Steinmeyer, Ortland & Jentsch, 1991*b*; Wischmeyer *et al.* 1993; Bardouille, Vullhorst & Jockusch, 1996). In contrast, no indication of hyperexcitability was observed in heterozygote adult carriers of the $Clc1^{adr}$ allele. For adult muscle, there are two explanations for recessiveness, i.e. complete phenotypic wild-type behaviour of mice carrying one functional (wild-type) and one defective allele of the *Clc1* gene. (1) The G_{Cl} could be proportional to the wild-type gene dosage, but 50% of wild-type G_{Cl} may still be sufficient for normal muscle physiology. This seems a reasonable

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hypothesis as pharmacological experiments have shown that lowering G_{Cl} to 30% of normal still does not lead to myotonia (Barchi, 1975). (2) There could be a post-transcriptional regulatory mechanism, active in the adult muscle fibres, that adjusts G_{Cl} to the required level irrespective of whether one or two copies of the functional *Clc1* gene are transcribed.

Our genetic and physiological data support the second hypothesis, thereby indicating a novel long-term regulatory mechanism for the adjustment of G_{Cl} in mammalian muscle.

A preliminary report of this work has been given (Chen, Niggeweg & Jockusch, 1995).

METHODS

Mutant mice

The origin of the A2G strain carrying the $Clc1^{adr}$ mutation (Watkins & Watts, 1984) and the SWR/J strain carrying the $Clc1^{adr\text{-}mto}$ allele (Heller, Eicher, Hallett & Sidman, 1982) is described in Gronemeier *et al.* (1994). The genotypes of $Clc1^{adr}?/+$ and $Clc1^{adr\text{-}mto}?/+$ individuals were determined by polymerase chain reaction (PCR) diagnosis (Schnülle *et al.* 1997). The detection of the $Clc1^{adr}$ allele was done by amplification of genomic DNA with a mouse intron 12-specific and an Etn transposon-specific primer. The $Clc1^{adr\text{-}mto}$ allele differs from the wild-type allele by two base substitutions (Gronemeier *et al.* 1994). A PCR product of genomic DNA covering these mutations can be distinguished by single strand conformational polymorphism (Schnülle *et al.* 1997). For physiological and biochemical analyses, mice were anaesthetized and killed by gradually increasing CO_2 concentration in accordance with German law for the protection of animals.

mRNA analysis

RNA extractions and Northern blots were done according to established methods as described in Klocke, Steinmeyer, Jentsch & Jockusch (1994). The cDNA probes used for Northern blot hybridizations were: mouse ClC-1 '5′9-1', a 940 base pair (bp) cDNA extending from nucleotide 383 to nucleotide 1323 (positions according to rat ClC-1 cDNA; Steinmeyer *et al.* 1991*b*); 18S rRNA, a 1500 bp fragment of 18S rRNA (American Type Culture Collection, Rockville, MD, USA); glyceraldehyde 3-phosphate dehydrogenase (GAPDH), full length cDNA (kindly provided by Dr Rolf Müller, University of Marburg, Germany). In one set of experiments, preflashed X-ray films were exposed to the ^{32}P-label of Northern blots (cf. Klocke *et al.* 1994) and were densitometrically scanned using a Hewlett-Packard ScanJet IIcx/T and the QuantiScan evaluation program from Biosoft (Cambridge, UK). Hybridization signals of ClC-1 mRNA were normalized using either the 18S rRNA or the GAPDH mRNA, or both signals obtained by a subsequent hybridization of the same filter. In more recent experiments, ^{32}P-hybridization signals (after localizing the radioactivity by autoradiography) were quantified with a bioimager (Fujix bas 1000; Fuji, Tokyo) and related to the signal of GAPDH mRNA on the same lane. The results obtained were identical to within ±15% with these two methods of quantification and the two standards used for normalization.

For conductance measurements, fibres from either the sternocostalis muscle or from the diaphragm were used. Ribcages were prepared from freshly killed mice, and sternocostalis fibres impaled through a window in a half ribcage. Tetrodotoxin (0·1–0·2 μM) and dantrolene sodium (3 mg ml^{-1}) (Sigma) were used to prevent contractions. Electrical connection with the bath was through an Ag–AgCl electrode. The current electrodes were filled with either 2 M potassium citrate (resistance 30–60 MΩ) or 3 M KCl (10–40 MΩ); the voltage electrode was filled with 3 M KCl (30–60 MΩ). Measurements were done at 37 °C in standard Ringer solution of composition (mM): NaCl, 140; $CaCl_2$, 2; $MgCl_2$, 1; KOH, 5; NaOH, 7; CH_3SO_3H, 12; glucose, 12; Hepes, 2. In chloride-free Ringer solution, Cl^- was replaced in equimolar quantities by substituting methylsulphate salts for NaCl and KCl and nitrate salts for $CaCl_2$ and $MgCl_2$. All solutions were gassed with 95% O_2–5% CO_2 (pH 7·3–7·4).

To determine conductances in sternocostalis fibres two-electrode current clamp mode (distances, 0·05 mm and 0·4 mm) was used (Conte-Camerino, Bryant & Mitolo-Chieppa, 1982). The current pulse was set to produce less than 10 mV potential changes at 50 μm distance between the electrodes. Total and component conductances were calculated using the equations given in Hodgkin & Rushton (1946), Fatt & Katz (1951) and Bryant (1969). The diameters of live muscle fibres were determined using Nomarski optics and an ocular micrometer immediately after the electrophysiological measurements. Measurements from the diaphragm were performed using the three microelectrode method (Lehmann-Horn, Küther, Ricker, Grafe, Ballanyi & Rüdel, 1987).

RESULTS

Levels of ClC-1 mRNAs

The determination of the concentrations of the allelic forms of ClC-1 mRNAs as a function of gene dosages (0, 1 or 2 of each allele) was based on the fact that the ClC-1 mRNA transcribed from the *adr* allele of the *Clc1* gene lacks the

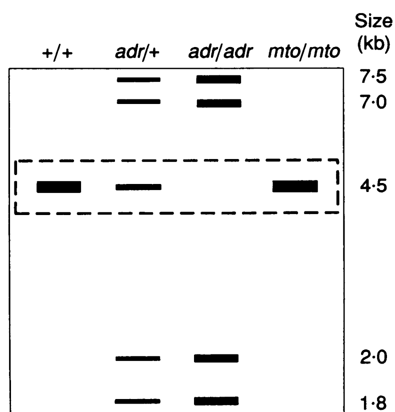

Figure 1. Allelic forms of ClC-1 mRNAs

Throughout this paper, alleles $Clc1^+$, $Clc1^{adr}$ and $Clc^{adr\text{-}mto}$ are abbreviated *wt* or +, *adr* and *mto*, respectively. Italics are used for gene and allele designations, roman letters for mRNAs (lower case) or phenotypes (upper case). Shown is a schematic representation of electrophoretic patterns (a Northern blot) of muscle RNAs from wild-type (+/+), heterozygous (*adr*/+), myotonic ADR (*adr*/*adr*), and myotonic MTO (*mto*/*mto*) mice. Functional wt and non-functional mto ClC-1 mRNAs are of the same size, 4·5 kb, but this size class is absent in the non-functional adr mRNA. Therefore, the wt and mto mRNAs can be quantified in the presence of adr mRNA by scanning the 4·5 kb band (indicated within dashed lines).

wild-type 4·5 kb size class and instead is represented by a doublet of higher and a doublet of lower chain lengths (Fig. 1; Steinmeyer *et al.* 1991*b*; Klocke *et al.* 1994). The former often produced variable signal intensities that were consistent within, but differed between, individuals. To correlate mRNA levels with function, i.e. G_{Cl}, levels of the wild-type mRNA in heterozygous animals were related to those in the homozygous wild-type state. Using for quantification either phosphoimaging on Northern blot filters (Fig. 2) or densitometry of X-ray films, the wild-type mRNA level in *adr*/+ animals was found to scatter around a mean value of about 50% of that in +/+ muscle (Klocke *et al.* 1994). With respect to this ratio, there was no difference between diaphragm, anterior tibial and vastus muscles. The adr-mto variant of mRNA cannot be distinguished from wild-type mRNA by Northern blotting (Fig. 1). However, two observations would argue that its expression is also proportional to gene dosage. In the compound heterozygote *adr*/*mto*, the level of adr-mto-type mRNA is the same as that of wild-type mRNA in *adr*/+ specimens. Furthermore, in *mto*/+ animals, the total signal is indistinguishable from that in *mto*/*mto* and in +/+ muscles. Hence, expression levels for each of the mRNA species, ClC-1 wild-type, adr and adr-mto, are roughly proportional to the dosage of the respective allele (Fig. 3).

Membrane conductances

G_m values were determined in fibres of the sternocostalis and diaphragm muscles. In accordance with the literature, mature adult muscles (> 120 days old) showed G_m values of

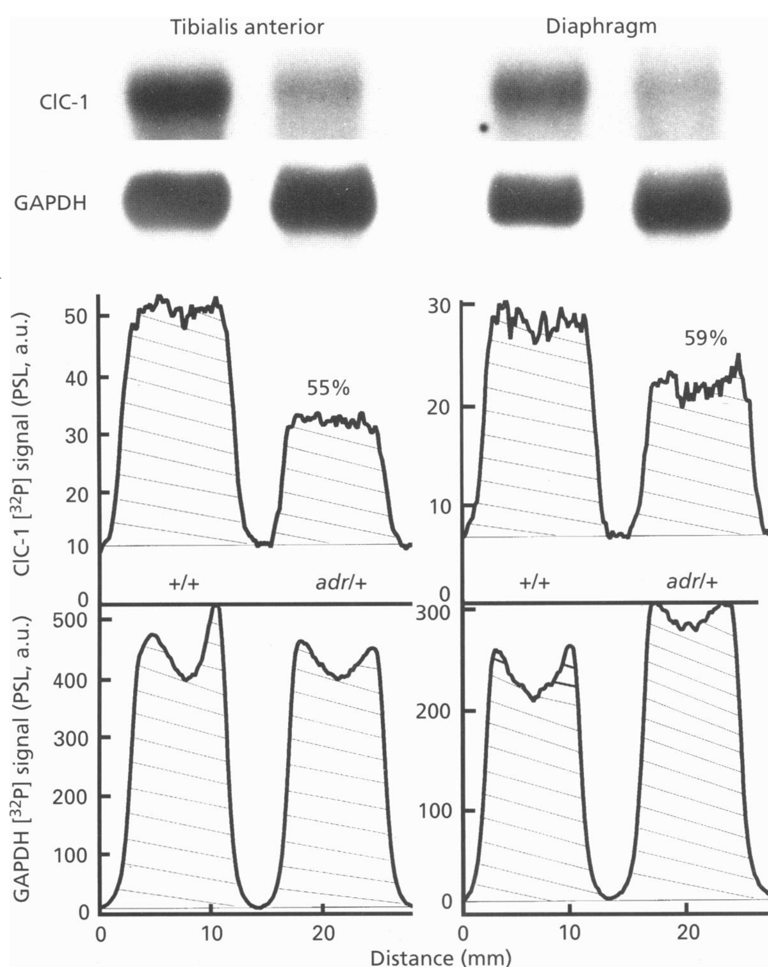

Figure 2. Determination of the concentration of functional ClC-1 mRNA in phenotypically normal mice with a homozygous wild-type (+/+) or heterozygous (*adr*/+) genotype

These mice are phenotypically indistinguishable. Upper panel, autoradiograph on X-ray film of an RNA blot hybridized with the ClC-1 probe; the 4·5 kb band of the wild-type ClC-1 mRNA is shown. Subsequently the same filter was hybridized with a GAPDH probe to yield the 1·2 kb band, the intensity of which was used as an internal reference for the quantification of the ClC-1 band. Lower panel, tracings perpendicular to the direction of electrophoresis (see Fig. 1) obtained directly from the radioactive filter with a bioimager. PSL, photostimulated luminescence in arbitrary units (a.u.) (note different absolute scales in left and right panels). The hatched areas between the tracing and the background level (i.e. the signal intensity in-between tracks) were used to calculate the normalized concentrations of ClC-1 mRNA (ClC-1 area/GAPDH area in same track); these are given as a percentage of wild-type +/+ values.

Table 1. Comparison of sarcolemmal G_m in young adult and fully mature heterozygous carriers of the myotonia gene *adr* with homozygous wild-type mice

Age (days)	Muscle	Genotype	n/m	G_m (μS cm^{-2})
60–80	Sternocostalis	+/+	36/8	1456 ± 91
		adr/+	37/5	1379 ± 71
>120	Sternocostalis	+/+	33/7	2323 ± 103
		adr/+	8/2	2133 ± 131
	Diaphragm	+/+	12/3	1958 ± 168
		adr/+	9/2	2227 ± 301

Two muscles appropriate for intracellular recordings, sternocostalis and diaphragm were compared in homozygous wild-type mice (+/+) and carriers of the $Clc1^{adr}$ myotonia allele (*adr*/+). n, number of fibres tested; m, number of muscles prepared. Fibres of the sternocostalis muscle increased in diameter from ~30 μm in 60- to 80-day-old mice to ~40 μm in mice older than 120 days, with no difference between +/+ and *adr*/+ specimens. Whereas G_m increased significantly between the young adult and the mature stages, there was no difference between homozygous wild-type and heterozygous individuals.

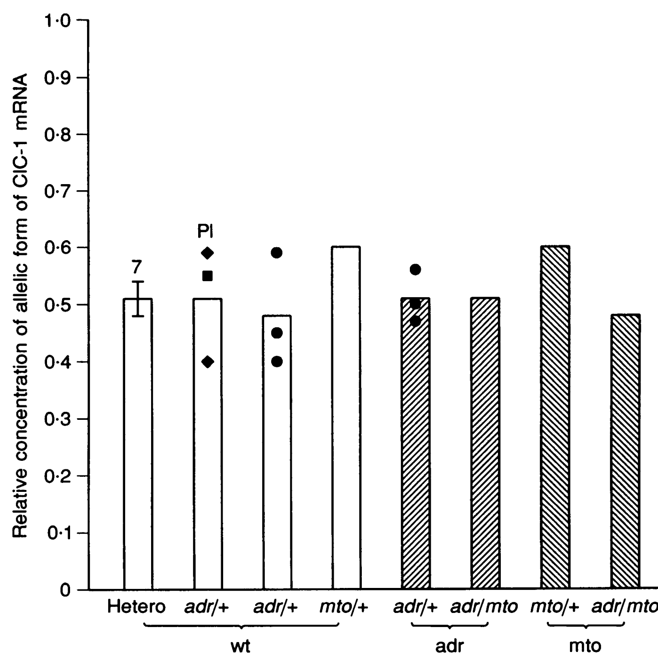

Figure 3. Levels of allelic forms of ClC-1 mRNAs relative to their level in the homozygous state in different muscles of mice with various genotypes

The allelic forms of ClC-1 mRNAs are given in roman letters, the genotypes of the mice in italics. Determinations shown in this figure were based on quantitative densitometry X-ray films exposed to Northern blots with 18S rRNA as a standard or by direct phosphoimaging (PI) of the filters (Fig. 2) with GAPDH as a standard. Wild-type (wt) ClC-1 RNA can be directly determined in the presence of adr mRNA, as the two have different size distributions; values for wt in *mto*/+ and mto in *mto*/+ were calculated under the assumption that the level of mto mRNA is equal in *adr*/*mto* and *mto*/*mto* mice. The values scatter around 0·5, indicating that the expression of individual allelic forms is proportional to gene dosage and independent of the respective other allele. Leftmost column, mean ± S.E.M. over all measurements made on functional wt mRNAs. Hetero, heterozygous *adr*/+ and *mto*/+. Symbols represent vastus (●), tibialis anterior (■) and diaphragm (◆).

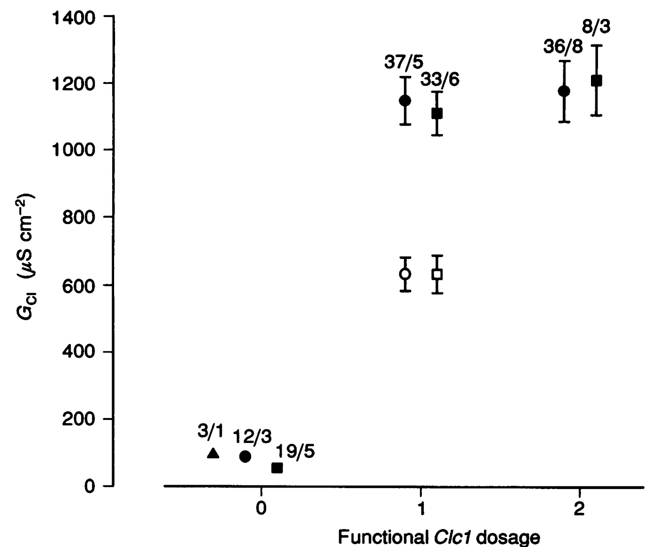

Figure 4. Sarcolemmal G_{Cl} of the sternocostalis muscles of 60- to 80-day-old mice carrying 0, 1 or 2 alleles of the myotonia mutations $Clc1^{adr}$ or $Clc1^{adr\text{-}mto}$

Only the homozygous mutant mice (*adr/adr* and *mto/mto*, functional gene dosage 0) and the compound heterozygote (*adr/mto*) showed overt myotonia. Values averaged over the *n/m* are given ± S.E.M. values (bars). Values for heterozygous animals (functional gene dosage 1) are not significantly different from those of homozygous wild-type animals (functional gene dosage 2) and significantly above the hypothetical calculated values for a halved G_{Cl} (open symbols), corrected by the residual G_{Cl} in the absence of functional *Clc1* gene. ●, *adr* and +; ■, *mto* and +; ▲, *adr/mto*.

~2000 μS cm^{-2} whereas with younger adults (60 to 80 days old) G_m of 1500 μS cm^{-2} was found. There was no difference between +/+ homozygous and *adr*/+ carrier phenotypic wild-type animals (Table 1).

In order to determine the main components of G_m, G_{Cl} (largely carried by ClC-1) and potassium conductance (G_K), measurements were done in the presence and absence of Cl^- ions.

Dependence on genotype

In confirmation of previous observations, G_{Cl} was found to be drastically diminished in myotonic ADR (Brinkmeier & Jockusch, 1987*a,b*; Mehrke *et al.* 1988) and MTO (Bryant, Mambrini & Entrikin, 1987) muscles. The same was true for the compound heterozygote $Clc1^{adr}/Clc1^{adr\text{-}mto}$ in accordance with its myotonic phenotype (Jockusch *et al.* 1988). In all these cases of myotonia, G_{Cl} was less than 10% of the wild-type level. In heterozygous animals, G_{Cl} levels were significantly above the calculated values for the expected contribution of the functional *Clc1* allele and not significantly different from those found in homozygous wild-type animals (Fig. 4). G_K values were confirmed to be lowered in myotonic muscle, again there was no difference between heterozygous muscle and homozygous wild-type control (Fig. 5).

DISCUSSION

In heterozygous carriers of recessive Becker type mutations of the chloride channel 1 gene, discharges have been detected by electromyography (Becker, 1977; Mailänder, Heine, Deymeer & Lehmann-Horn, 1996), indicating haplo-insufficiency with respect to the functional chloride channel gene. We have made similar observations on neonatal mice, at a time when the concentration of ClC-1 mRNA is still only 10–20% of that in adult mice (Wischmeyer *et al.* 1993; Bardouille *et al.* 1996). In contrast, our observations on adult carriers of myotonia mutations in the mouse not only indicate wild-type performance of muscle, but also a normal

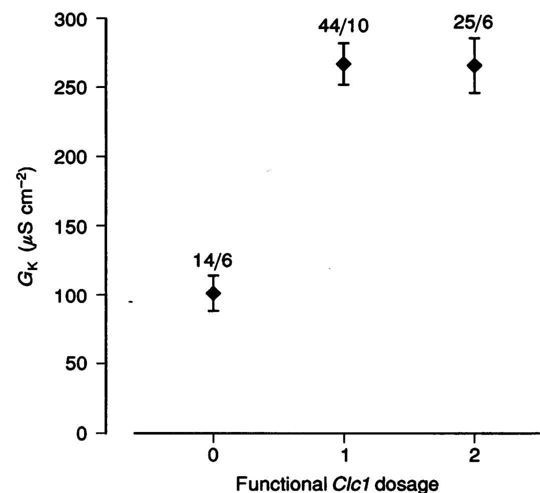

Figure 5. G_K of sternocostalis fibres as a function of allele dosages of the *Clc1* gene

Pooled values from *adr* and *mto* mutations. Calculations as in Fig. 4.

sarcolemmal chloride conductance. Our measurements were done on young adult mice (60–80 days old) at a developmental stage where the muscle fibres are fully differentiated, but G_m is still rising. Thus, *Clc1* gene dosage effects on G_m or G_{Cl}, if present in adult muscles, should be readily detectable.

For technical reasons, the anatomical origin of muscles used for mRNA analyses was not in all cases identical to those used for intracellular recording. However, both measurements were done on the diaphragm. In the rat, there is a dependence of ClC-1 mRNA levels on the type of muscle, fast or slow (Klocke *et al.* 1994), but in the mouse, muscles are predominantly fast and heterogeneous only with respect to oxidative or glycolytic metabolism, and this leads to a reduction of fibre type-specific differences among different muscles in comparison with the rat. In the wild-type mouse, the level of ClC-1 mRNA is regulated during development and by muscle activity: juvenile, slow and denervated muscles have lower levels than fast muscles (Wischmeyer *et al.* 1993; Klocke *et al.* 1994). However, in myotonic mice, despite the aberrant activity pattern of muscle and secondary downregulation of several genes (Jockusch, 1990), ClC-1 mRNA levels are similar to those in wild-type mice (Klocke *et al.* 1994). This fact has simplified the determination of mRNA levels for the *mto* allele. In all our data, there is no indication that the *relative* expression of the two alleles in heterozygous muscles would depend on the type of muscle; no mechanism for a non-random preference for the transcription of one allele of a gene is known.

There was no physiological difference between the heterozygous (+/defective) and the homozygous (+/+) state despite a considerable difference (factor of two) in the level of functional ClC-1 mRNA, i.e. in the absence of any compensation at the mRNA level. It follows that in adult muscle, gene dosage compensation must act at the post-transcriptional, presumably at the protein, level. Two mechanisms could be envisaged to explain this phenomenon. (1) There could be some additional protein present in limiting amounts with which ClC-1 subunits have to interact physically to form functional channels. This protein would have to be rather ubiquitous as functional ClC-1 channels have been obtained upon injection of only the ClC-1 message into non-muscle cells such as *Xenopus* oocytes (Steinmeyer *et al.* 1991*b*) or a human embryonic kidney cell line (Pusch, Steinmeyer & Jentsch, 1994). (2) Alternatively, some mechanisms regulating and limiting channel density in the plasmalemma itself could be responsible for the 'ceiling' of G_{Cl}. These hypotheses could be tested by appropriate transfection experiments using myogenic cells as recipients.

So far, there is only one known mechanism for the acute regulation of G_{Cl}: protein kinase C (PKC)-catalysed phosphorylation of ClC-1 or an associated protein (Brinkmeier & Jockusch, 1987*b*). As shown by the application of inhibitors of PKC, this regulation is not responsible for the low G_{Cl} in the myotonic mouse (Brinkmeier & Jockusch, 1990) and seems irrelevant for the adjustment of G_{Cl} in mature muscle. Our present results call for the investigation of a long-term mechanism that regulates muscular chloride channels in such a way that their overall activity, G_{Cl}, is limited to a value which may depend on the type of skeletal muscle, slow or fast, glycolytic or oxidative.

The nature of the downregulation of G_K, e.g. which K^+ channel types are affected in myotonic muscle, is still unknown. Because K^+ channel genes involved are independent of the *Clc1* locus, this is probably a secondary long-term consequence of myotonia, as has been observed for many other features of the muscle fibre, e.g. parvalbumin levels or oxidative–glycolytic metabolism (for review see Jockusch, 1990). The normal level of G_K in heterozygotes would then appear as a consequence of the normal physiological activity of muscle.

The greater than twofold excess in adult mouse muscle of ClC-1 mRNA over the concentration required to synthesize saturating quantities of ClC-1 channel protein may be a safeguard mechanism specific for small mammals. Their skeletal muscles contract and relax much faster than those of large mammals. Correspondingly, haplo-insufficiency (indicating lack of a ClC-1 safeguard mechanism) is observed in humans carrying only one recessive myotonia allele (Becker, 1977; Mailänder *et al.* 1996), and in neonate mice but not in adult mice. A less stringent requirement for CLC-1 in human as compared to murine skeletal muscle is reflected in the fact that Becker- and Thomsen-type myotonia patients are affected to a much lesser extent than myotonic mice.

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